



# Characterization of a dual-tropic Human immunodeficiency virus (HIV-1) strain derived from the prototypical X4 isolate HXBc2

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## ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) coreceptor usage and tropism can be modulated by the V3 loop sequence of the gp120 exterior envelope glycoprotein. For coreceptors, R5 viruses use CCR5, X4 viruses use CXCR4, and dual-tropic (R5X4) viruses use either CCR5 or CXCR4. To understand the requirements for dual tropism, we derived and analyzed a dual-tropic variant of an X4 virus. Changes in the V3 base, which allow gp120 to interact with the tyrosine-sulfated CCR5 N-terminus, and deletion of residues 310/311 in the V3 tip were necessary for efficient CCR5 binding and utilization. Thus, both sets of V3 changes allowed CCR5 utilization with retention of the ability to use CXCR4. We also found that the stable association of gp120 with the trimeric envelope glycoprotein complex in R5X4 viruses, as in X4 viruses, is less sensitive to V3 loop changes than gp120-trimer association in R5 viruses.

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## Introduction

The entry of human immunodeficiency virus type 1 (HIV-1) into the host cell is mediated by the viral envelope glycoproteins (Choe et al., 1998; Wyatt and Sodroski, 1998). The envelope glycoproteins, gp120 (SU) and gp41 (TM), constitute a trimeric complex that is anchored on the virion surface by the membrane-spanning segments of gp41 (Chan et al., 1997; Farzan et al., 1998; Weissenhorn et al., 1997; Zhu et al., 2003). The mature envelope glycoproteins form a trimer in which three gp120 subunits are noncovalently bound to three membrane-anchored gp41 subunits (Helseth et al., 1991). The initial binding of gp120 to the cellular receptor CD4 triggers conformational changes in gp120 that allow the subsequent interaction with one of the chemokine coreceptors, usually CCR5 or CXCR4

(Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng et al., 1996; Wu et al., 1996). Coreceptor binding is thought to induce additional conformational changes in the envelope glycoproteins that lead to the fusion of the viral and target cell membrane (Hoffman and Doms, 1999).

HIV-1 can be classified into three phenotypes based on the virus' ability to use the CCR5 and/or CXCR4 coreceptor (Berger et al., 1998). R5 viruses use CCR5 as the coreceptor, X4 viruses use CXCR4 as the coreceptor and R5X4 (dual-tropic) viruses can use both coreceptors. HIV-1 primarily infects human CD4-positive T cells and macrophages. Cellular tropism can be determined by coreceptor usage (Rana et al., 1997). R5 viruses infect primary macrophages and T lymphocytes, whereas X4 viruses infect primary T lymphocytes and T-cell lines (Rana et al., 1997). The coreceptor usage, and thus, cellular tropism, is mainly determined by the third variable loop (V3 loop) of the gp120 exterior envelope glycoprotein (Chavda et al., 1994; Chesebro et al., 1996; Hwang et al., 1991).

The V3 loop of HIV-1 gp120 is about 34–37 residues in length but exhibits significant variability among different isolates (Hartley et al., 2005). Structurally, the V3 loop can be divided into three regions: the base, the stem and the tip (crown) (Huang

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et al., 2005). The V3 stem is more variable in sequence, whereas the base and tip are relatively conserved. Because the V3 loop is the main determinant of coreceptor usage (Chesebro et al., 1996; Hoffman et al., 2002; Shioda, Levy, and Cheng-Mayer, 1992; Willey, Theodore, and Martin, 1994), it has been intensively studied for the purposes of understanding interactions with the coreceptors and predicting coreceptor usage of HIV-1 isolates. In general, the V3 loops of X4 viruses have a greater number of positive charges than those of R5 viruses (Jensen et al., 2003; Low et al., 2007); however, distinct sequence characteristics have not been defined for the V3 loops of dual-tropic viruses. Some studies have shown that residues 306, 321 and 322, the N-linked glycan at residue 301, and the total number of positive charges in the V3 loop are important for determining coreceptor preference (Cardozo et al., 2007; de Jong et al., 1992; Fouchier et al., 1995; Ogert et al., 2001; Polzer et al., 2002). In addition, some bioinformatics tools have been developed to predict coreceptor usage (Chueca et al., 2009; Jensen et al., 2003). However, the prediction of coreceptor usage for a given V3 loop based only on the V3 amino acid sequence is still imperfect.

In this report, we explore in more detail the interactions between the V3 loop sequences and coreceptors CCR5 and CXCR4. We identify an interesting derivative of the prototypic X4 strain, HXBc2, that has acquired the ability to use CCR5 but still retains CXCR4 usage. Two residues in the base of the V3 loop were found to be critical for this dual-tropic phenotype. Modeling based on available x-ray crystal and NMR structures and mutagenesis data suggest that these residues contact the tyrosine-sulfated N-terminus of the chemokine receptor. Another pair of HXBc2 amino acid residues at the tip of the V3 loop was found to be detrimental to CCR5 binding and had to be deleted to allow CCR5 tropism. In addition to analyzing the effects of these changes on HIV-1 tropism, we examined the impact of the V3 changes on envelope glycoprotein trimer stability and discovered that R5X4 and X4 HIV-1 exhibit similar phenotypes that are distinct from those of R5 HIV-1.

Results

Generation of a dual-tropic HXBc2 variant.

We wished to study the specific role of certain residues in the gp120 V3 loop that are important for coreceptor interaction, with the aim of understanding the mechanism by which some HIV-1 viruses acquire dual tropism. Fig. 1 shows an alignment of the V3 loops of some common X4-, R5-, and dual-tropic viruses. Most natural HIV-1 isolates lack residues 310–311 (QR in some laboratory-adapted X4-tropic viruses). Residues 325 and 326 (NM in the laboratory-adapted X4-tropic viruses) are more commonly DI in primary R5 or R5X4 HIV-1 strains, suggesting that these residues could be important for CCR5 interaction. Previous studies have shown the importance of some of these

motifs in virus tropism (Hung et al., 1999); however, the exact mechanism by which these changes affect viral phenotypes has not been defined. To investigate the contribution of these residues to coreceptor choice, we generated HXBc2 mutants containing a deletion in residues 310–311 (ΔQR) and/or the changes N325D/M326I (DI) (Fig. 2A). The infectivity of these V3 loop mutants was analyzed in Cf2Th cells expressing CD4 and either CXCR4 or CCR5. Corroborating the hypothesized role of these residues in coreceptor usage, we observed that, like the R5-tropic ADA virus and the dual-tropic 89.6 virus, the DIΔQR mutant was able to infect Cf2Th cells expressing CD4 and CCR5 efficiently (Fig. 2B). Surprisingly, the DIΔQR mutant retained the ability to infect CD4/CXCR4 cells efficiently and thus exhibited dual tropism.

To characterize further the contribution of the two V3 elements to tropism, we generated HXBc2 viruses containing only the DI substitutions (DI) or the QR deletion (ΔQR). When these changes were individually introduced, the resulting mutant viruses were unable to mediate entry of HIV-1 into CD4/CCR5 cells. In addition, their infectivity in CD4/CXCR4 cells was reduced compared to that of the wild-type HXBc2 (27% and 68% for the DI and ΔQR mutants, respectively) (Fig. 2B). The substitution of an alanine residue for Arg 311 in the context of the DI mutant (DI/R311A) abolished the infectivity in CD4/CCR5 and in CD4/CXCR4 cells (Fig. 2B). By contrast, changing Arg 306 to Ser did not alter coreceptor usage in the context of either HXBc2 or the DI mutant (Fig. 2B). Together, these data suggest that both the ΔQR and DI changes in the V3 region are necessary for the dual-tropic phenotype.

Binding of DIAQR mutants to the 412d antibody and CCR5.

The 412d monoclonal antibody mimics the tyrosine sulfate-containing N-terminus of the CCR5 coreceptor (Choe et al., 2003; Huang et al., 2007; Xiang et al., 2005). To assess the binding of the V3 loop mutants to the 412d antibody, cell supernatants containing radiolabeled gp120 glycoproteins were immunoprecipitated with 412d and the bound gp120 was analyzed on an SDS-PAGE gel. As expected, the wild-type HXBc2 gp120 and the X4 ΔQR and R306S mutants did not efficiently bind the 412d antibody (Fig. 2C). However, 412d was able to bind and immunoprecipitate the R5X4 DIAQR mutant. Surprisingly, 412d also recognized the DI and DI/R306S mutants (Fig. 2C), even though these two mutants were not able to infect CD4/CCR5 cells. Similar results were obtained when the DI change was introduced into the gp120 glycoprotein of another X4 virus, NL4-3 (Fig. 2C). These results indicate that the DI change, but not the ΔQR deletion, is required for 412d antibody binding.

The binding of the V3 loop mutants to CCR5 was also studied. Supernatants from cells expressing radiolabeled gp120 were incubated, in the presence of soluble CD4, with cells expressing CCR5. The bound gp120 glycoprotein was analyzed by SDS-PAGE. In contrast with the 412d binding results, when we tested the DI

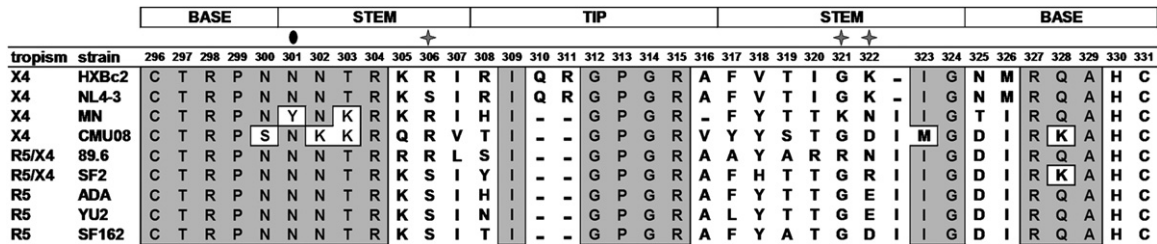
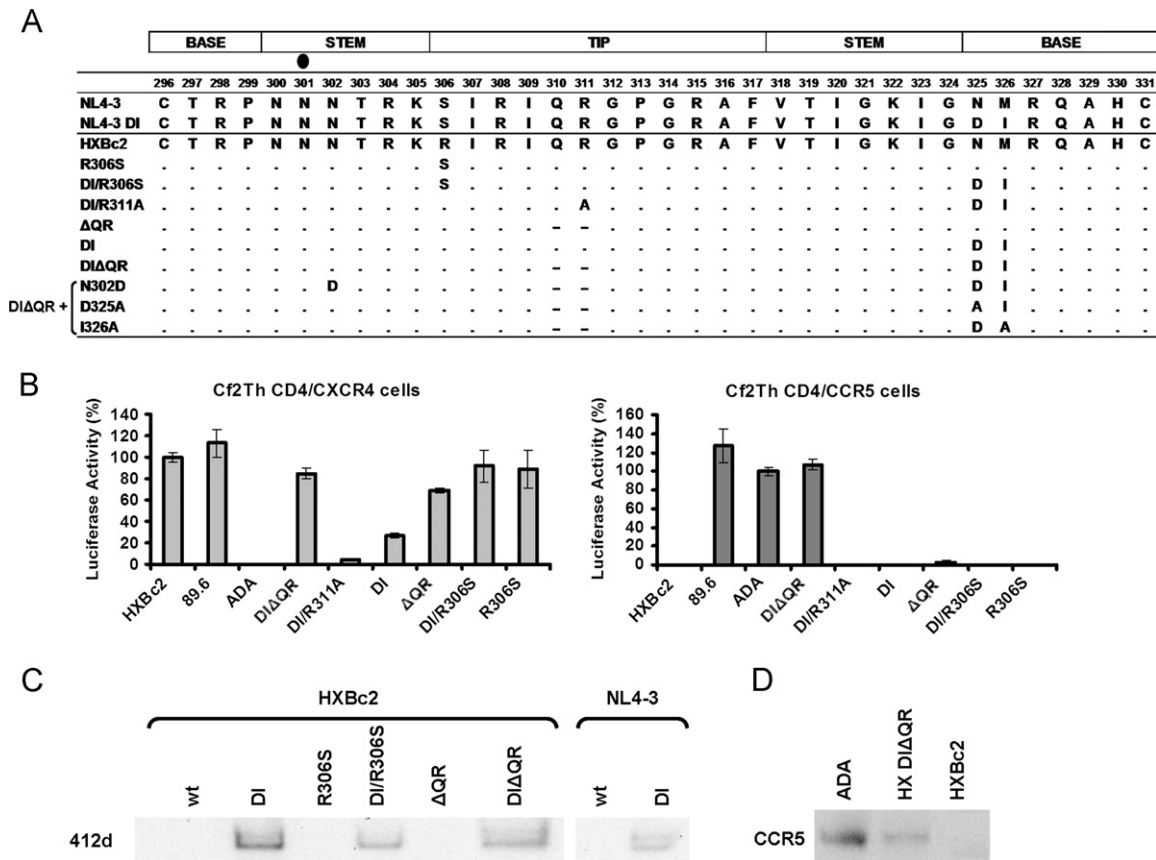


Fig. 1. Sequence alignment of the V3 loops of several common X4-, R5-, and dual-tropic HIV-1 strains. Amino acid position numbering is based on that of the prototypic HXBc2 strain, as per current recommendations (Korber et al., 1998). Key V3 residues that have been previously implicated in coreceptor choice are highlighted with stars. Residues conserved among the V3 loops shown are boxed. A potential N-linked glycosylation site at asparagine 301 is indicated by a black oval.

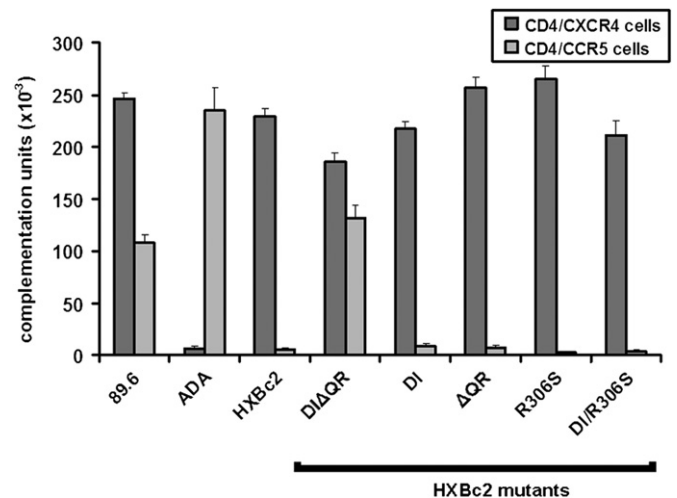


**Fig. 2.** Sequences and phenotypes of V3 variants of X4 envelope glycoproteins. **(A)** Sequence alignment of the V3 loops of the HXBc2 and NL4-3 mutants used in this study. A dot indicates the same residue and a dash a deletion. The potential N-linked glycosylation site at asparagine 301 is labeled with a black oval. **(B)** Infectivity of HXBc2 V3 loop mutants in cells expressing CD4 and either CXCR4 or CCR5. HIV-1 vectors encoding luciferase and pseudotyped with the indicated envelope glycoproteins were incubated with cells. Two days later the cells were lysed and assayed for luciferase activity. The results shown are representative of those obtained in three independent experiments. The values shown are the means of four data points from a single experiment and the error bars represent the standard deviations. **(C)** Binding of HXBc2 or NL4-3 gp120 mutants with changes in the V3 loop to the CCR5-mimetic 412d monoclonal antibody. 293T cells expressing the wild-type (wt) or V3 loop mutant HIV-1 envelope glycoproteins were radiolabeled. The cell supernatants were precipitated by the 412d monoclonal antibody. The gp120 glycoproteins precipitated from the media were analyzed by SDS-PAGE and autoradiography. **(D)** Binding of wt and mutant gp120 glycoproteins to CCR5. The indicated radiolabeled gp120 envelope glycoproteins were incubated at room temperature for 1 h with Cf2Th-CCR5 cells in the presence of sCD4. The cells were washed and lysed, and the bound gp120 was precipitated by a mixture of sera from HIV-1-infected individuals. The bound gp120 glycoprotein was analyzed by SDS-PAGE and autoradiography.

and DI/R306S mutants for their ability to bind CCR5, we observed that the DI change alone was not sufficient to allow binding to CCR5 (data not shown); only the DIΔQR mutant was able to bind CCR5 detectably (Fig. 2D). Apparently, the DI change is sufficient to allow binding to 412d and perhaps, by analogy, with the tyrosine-sulfated CCR5 N-terminus; however, other changes, specifically the removal of the QR residues in the V3 tip, are required for binding to the intact CCR5 coreceptor. These binding results explain why only the DIΔQR mutant, but not the DI mutant, is able to infect CD4/CCR5-expressing cells.

#### Membrane fusion ability of the DIΔQR mutant.

The ability of the V3 loop mutants to mediate the fusion of envelope glycoprotein-expressing cells and cells bearing CD4 and either CCR5 or CXCR4 was assessed using the  $\alpha$ -complementation assay (Holland et al., 2004). 293 T cells were cotransfected with plasmids expressing the mutant envelope glycoproteins and the  $\alpha$ -fragment of  $\beta$ -galactosidase. The cotransfected 293 T cells were cocultivated with canine Cf2Th cells expressing CD4, one of the coreceptors CCR5 or CXCR4, and the  $\omega$  fragment of  $\beta$ -galactosidase. The fusion of the envelope-expressing 293 T cells with the Cf2Th cells expressing HIV-1 receptors results in the reconstitution of enzymatically active  $\beta$ -galactosidase. The measurement of



**Fig. 3.** Syncytium-forming activity of HIV-1 envelope glycoprotein variants. 293T cells expressing the indicated HIV-1 envelope glycoproteins were cocultivated with Cf2Th-CD4/CCR5 cells and syncytium formation measured as described in Materials and methods. The values shown are the means of six data points from a single representative experiment and the error bars represent the standard deviations.

$\beta$ -galactosidase activity provides a quantification of the extent of the fusion. The membrane fusion data (Fig. 3) showed that all the mutants assayed, DI,  $\Delta$ QR, R306S, DI/R306S and D $\Delta$ QR, retained the ability to fuse with CD4/CXCR4-expressing cells at levels comparable to that of the wild-type HXBc2 envelope glycoproteins. Only the D $\Delta$ QR mutant gained the ability to fuse with CD4/CCR5-expressing cells at levels comparable to that of the envelope glycoproteins of the dual-tropic virus 89.6. Thus, the results of the cell–cell fusion assay agree with the infectivity and CCR5 binding data (Fig. 2).

*The DI motif in the C-terminal region of the V3 loop is necessary for CCR5 usage.*

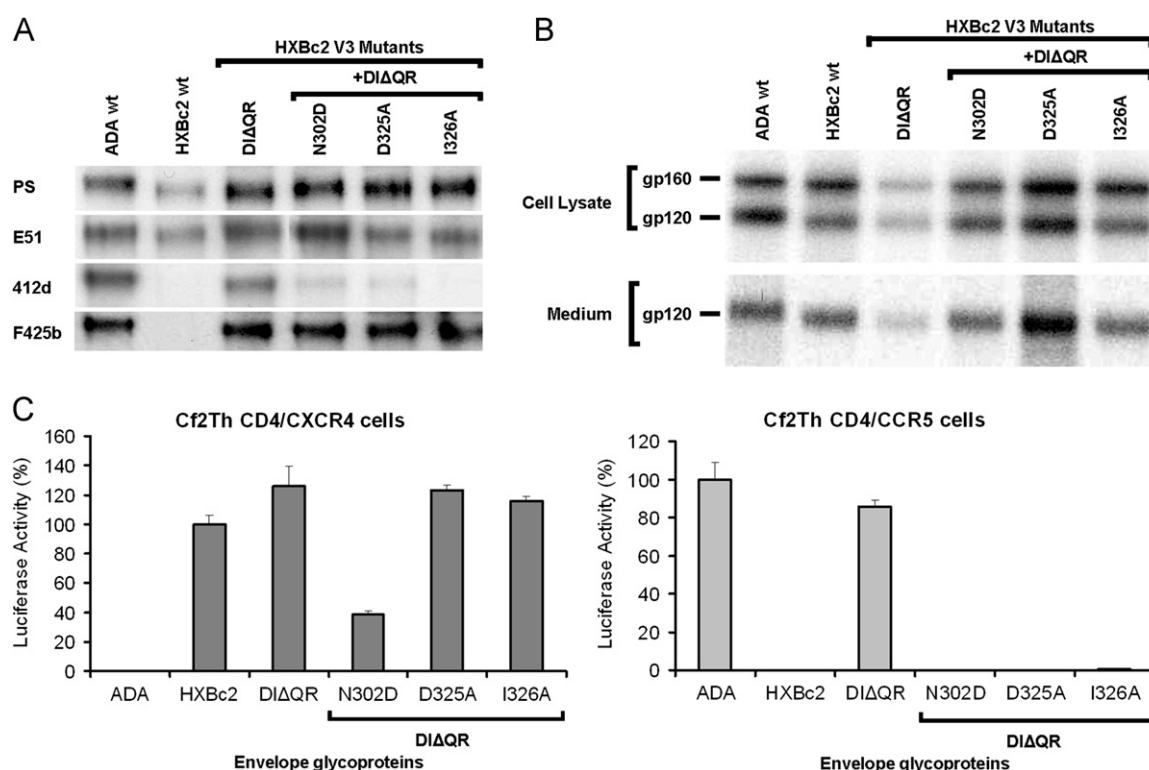
The above results indicated that, in the context of HXBc2 envelope glycoprotein, the DI motif (residues 325–326) in the C-terminal segment of the V3 loop is important for CCR5 usage and binding (Fig. 2B and D), as well as sufficient for 412d binding (Fig. 2C). Thus, we further dissected this motif by making single alanine substitutions in residues 325 and 326 of the D $\Delta$ QR mutant (Fig. 2A). The binding of the resulting mutants, D $\Delta$ QR D325A and D $\Delta$ QR I326A, to the 412d antibody was significantly reduced or abolished, respectively (Fig. 4A). We also tested the binding of these mutants to a mixture of polyclonal sera from HIV-1-infected individuals and monoclonal antibodies E51, a CD4-induced antibody, and F425b, an antibody that specifically binds to the V3 loop of R5 viruses (Cavacini et al., 2003; Xiang et al., 2003). The 412d and F425b antibodies precipitated the R5

ADA gp120 but not the X4 HXBc2 gp120, as expected. Both D $\Delta$ QR mutants were recognized by the E51 and F425b antibodies; the D $\Delta$ QR D325A and D $\Delta$ QR I326A mutants were precipitated slightly less efficiently than D $\Delta$ QR by the E51 antibody (Fig. 4A). Both mutant envelope glycoproteins were processed at levels comparable to wild-type HXBc2 and ADA envelopes (Fig. 4B). The ratio of gp120 shed into the medium relative to the cell-associated gp120 was similar to that of the wild-type glycoproteins for all the mutants (Fig. 4B). These results together indicate that the reduced binding to 412d was not due to a global misfolding of the mutant envelope glycoproteins, and that aspartic acid 325 and isoleucine 326 are important for the recognition of gp120 by the 412d antibody.

The infectivity of the D325A and I326A mutants was assessed in Cf2Th cell lines expressing the HIV-1 receptors. Both mutants infected CD4/CXCR4 cells efficiently (Fig. 4C), suggesting that these mutant envelope glycoproteins are properly folded and functional for CXCR4 binding. However, the D325A and I326A changes resulted in a loss of the ability of the D $\Delta$ QR mutant to infect CD4/CCR5 cells (Fig. 4C). These results support the importance of the DI motif for CCR5 coreceptor usage, but not for CXCR4 usage.

*Asparagine 302 in the V3 N-terminus contributes to CCR5 binding*

In the N-terminal segment of the V3 loop there is a motif of three asparagines (NNN) that is relatively well conserved among most HIV-1 strains (Fig. 1). This motif lies in the base of the V3 loop in close proximity to residues 325–326 (Huang et al., 2007;



**Fig. 4.** Phenotypes of HXBc2 D $\Delta$ QR envelope glycoproteins with changes in the V3 base. (A) Binding of ligands to HIV-1 envelope glycoprotein variants. 293T cells expressing wild-type (wt) or V3 loop mutant HIV-1 envelope glycoproteins were radiolabeled. The cell supernatants were precipitated by a mixture of sera from HIV-1-infected individuals (PS) or by the indicated monoclonal antibody. The gp120 glycoproteins precipitated from the media were analyzed by SDS-PAGE and autoradiography. (B) Processing and subunit association of HIV-1 envelope glycoprotein V3 loop mutants. 293T cells expressing wild-type (wt) or mutant envelope glycoproteins from the indicated HIV-1 strains were radiolabeled. The cells were pelleted and lysed. The radiolabeled cell lysates and media were precipitated by a mixture of sera from HIV-1-infected individuals. The envelope glycoproteins precipitated from the cell lysates and media were analyzed by SDS-PAGE. The gp160 and gp120 envelope glycoproteins are indicated. (C) Infectivity of HXBc2 V3 loop mutants in cells expressing CD4 and either CXCR4 or CCR5. HIV-1 vectors encoding luciferase and pseudotyped with the indicated envelope glycoproteins were incubated with cells. Two days later the cells were lysed and assayed for luciferase activity. The results shown are representative of those obtained in two independent experiments. The values shown are the means of four data points from a single experiment and the error bars represent the standard deviations.



Schnur et al., 2011). We investigated the importance of this region to CCR5 and CXCR4 usage by changing Asn 302 to aspartic acid; this change does not delete the potential N-linked glycosylation site at Asn 301. Introduction of an N302D change in the context of the D1ΔQR envelope glycoprotein drastically reduced the binding of gp120 to the 412d antibody (Fig. 4A), as was seen for the D325A and I326A mutants. The D1ΔQR N302D mutant was recognized efficiently by the E51 and F425b monoclonal antibodies. To confirm whether the N302D change would affect the coreceptor usage of the D1ΔQR virus, we tested the ability of this mutant to infect cells expressing CD4 and either CCR5 or CXCR4. The expression, processing and subunit association of the D1ΔQR N302D mutant were comparable to those of the wild-type HXBc2 envelope glycoproteins and the D1ΔQR mutant (Fig. 4B). However, the N302D change abolished the ability of the D1ΔQR envelope glycoproteins to infect CD4/CCR5 cells (Fig. 4C) and reduced by about 3-fold the infection of CD4/CXCR4 cells. Thus, the relatively conservative substitution of aspartic acid for asparagine 302 resulted in disruption of CCR5 usage.

#### *Changes in the V3 N-terminus of primary virus envelope glycoproteins*

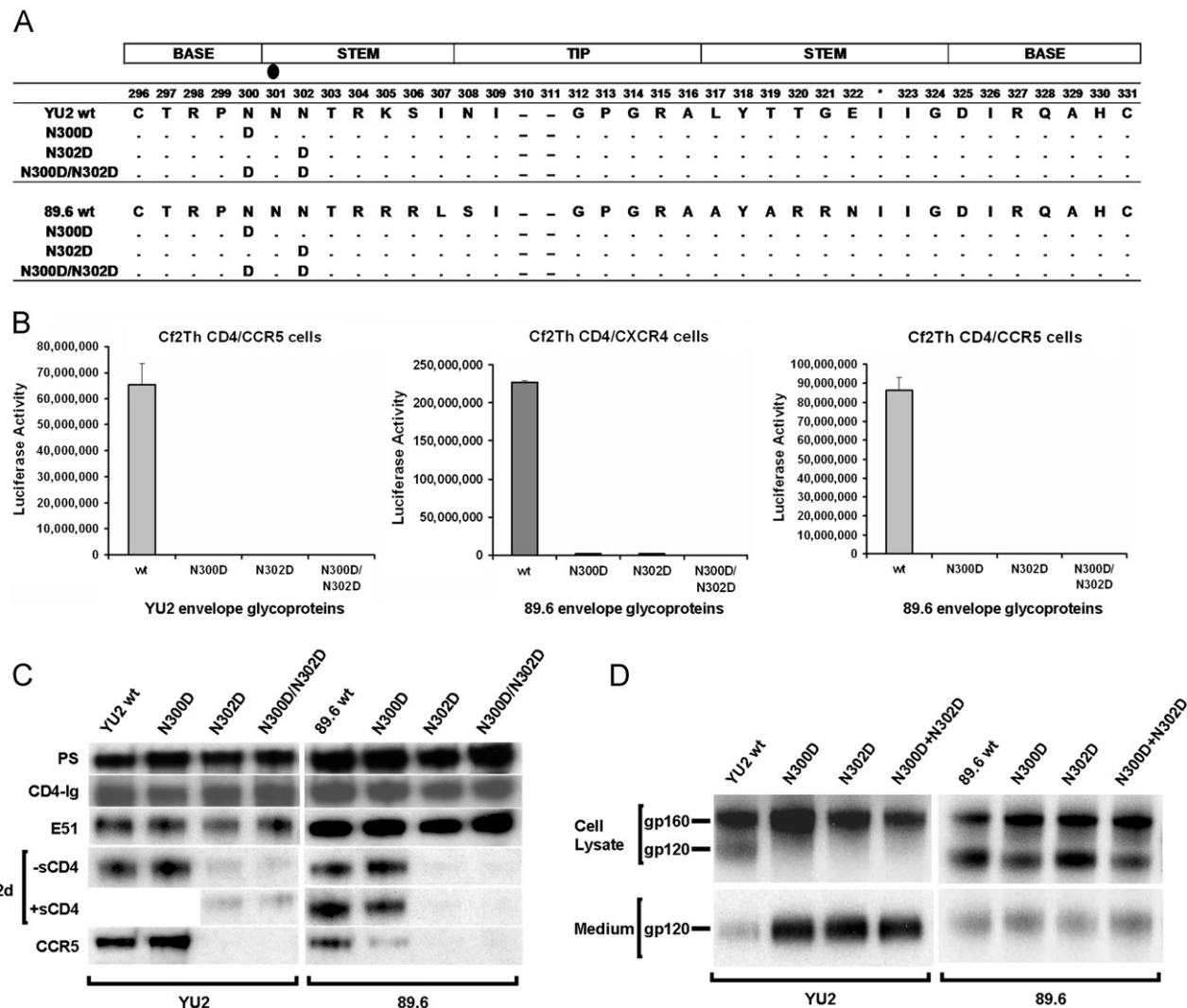
To investigate the Asn-rich region in the N-terminus of the gp120 V3 region of natural R5 or R5X4 viruses, we introduced changes in residues Asn 300 and Asn 302 of the YU2 (R5) and 89.6 (R5X4) envelope glycoproteins (Fig. 5A). Asn 301 is an N-linked glycosylation site and the elimination of this glycan could significantly modify the conformation of this region, so this residue was not altered. The introduction of the changes, N300D and N302D, alone or in combination, in the dual-tropic 89.6 strain eliminated its infectivity in Cf2Th cells expressing CD4 and CCR5 or CD4 and CXCR4 (Fig. 5B). Similarly, these changes also eliminated the infectivity of viruses with the YU2 envelope glycoproteins in Cf2Th cells expressing CD4 and CCR5 (Fig. 5B). To examine whether these changes might affect the integrity of the envelope glycoproteins, we examined the binding of these mutants to several molecules (Fig. 5C). Binding of all the mutants to CD4-Ig and the E51 antibody was efficient and comparable to that of wild-type 89.6 and YU2 gp120; however, the binding of mutants N302D and N300D/N302D to the 412d antibody and CCR5 was drastically reduced, which may have contributed to the lack of infectivity of these mutants in CD4/CCR5 cells. The N300D change did not affect the binding to the 412d antibody for the gp120 glycoprotein of either strain. This change significantly reduced the binding of 89.6 gp120 to CCR5, helping to explain the lack of infectivity of this mutant. The YU2 N300D mutant efficiently binds CCR5 (Fig. 5C), even though it does not support infection of CD4/CCR5 cells (Fig. 5B). These results suggested that different mechanisms might contribute to the loss of infectivity of the 89.6 and YU2 mutants. To investigate this possibility, we studied the expression, processing and subunit association of these envelope glycoproteins. Although the expression levels and processing of the mutants were comparable to those of the wild-type envelope glycoproteins, all the YU2 mutants exhibited dramatic shedding of gp120 into the medium (Fig. 5D). By contrast, the expression, processing and subunit association of the 89.6 mutants were comparable to those of the wild-type envelope glycoproteins. These data suggest that the lack of infectivity of the N300D and N302D YU2 mutants is due to the instability of gp120 association with the envelope glycoprotein trimer rather than an effect on CCR5 binding ability.

## **Discussion**

Many previous studies have demonstrated the role of the HIV-1 gp120 V3 loop in coreceptor binding and determination of R5 or

X4 tropism (Cardozo et al., 2007; Foda et al., 2001; Maeda et al., 2008a; Sander et al., 2007; Svicher et al., Perno; Verrier et al., 1999; Xiao et al., 1998). Here, we addressed how a single V3 structure in a dual-tropic virus meets the requirements to recognize the CCR5 and CXCR4 coreceptors. By systematically studying the contribution of V3 loop changes to the generation of an R5X4 variant, D1ΔQR, from the prototypical X4 virus HXBc2, we can suggest a reasonable mechanistic model for dual tropism. First, our studies underscore the critical importance of sequences in the base of the V3 loop for coreceptor binding. The N-terminus and C-terminus of the V3 loop, which constitute the V3 base, are quite conserved among X4 and R5 viruses. However, our results show that the DI motif at residues 325 and 326 in the C-terminal region of the V3 loop is important for CCR5 utilization as well as for binding to the 412d antibody, but plays a less important role in CXCR4 usage. The heavy chain complementarity-determining region (CDR) loop of the 412 antibody has sulfate-modified tyrosine residues that mimic the CCR5 N-terminus (Choe et al., 2003; Dorfman et al., 2006; Huang et al., 2007; Xiang et al., 2005). For example, it has been shown that these 412d sequences can functionally replace the CCR5 N-terminus to allow coreceptor function for R5 HIV-1 entry (Xiang et al., 2005). These results suggest that Asp 325 and Ile 326 might be directly interacting with CCR5, but not with CXCR4. Although the full structure of CCR5 has not been solved, the NMR structure of a CCR5 N-terminal peptide in the presence or absence of gp120 has been determined and provides some insight into the interactions between gp120 and the N-terminus of CCR5 (Huang et al., 2007; Schnur et al., 2011). The docking model of gp120 and the CCR5 N-terminal peptide (Fig. 6A) predicts specific interactions between the N-terminus of CCR5 and the base of the gp120 V3 loop. In this model, gp120 residues Asn 302, Asp 325 and Ile 326 interact with Asn 13 and the key sulfated Tyr 10 and Tyr 14 residues in the CCR5 N-terminus. In the crystal structure of the HIV-1 gp120 core in complex with CD4 and the Fab fragment of the CCR5-mimetic 412d antibody, the 412d antibody makes similar contacts with the gp120 V3 loop (Huang et al., 2007). The CDR3 loop of the 412d antibody heavy chain crosses over the base of the gp120 V3 loop and the sulfated tyrosines Tys 100 and Tys 100c of 412d closely interact with gp120 residues N302, D325 and I326 (Fig. 6B). Antibody 412d, however, interacts with a much larger overall surface of gp120 than the CCR5 N-terminal peptide (Huang et al., 2007). The additional contacts made by the 412d antibody with gp120 residues conserved among R5 and X4 viruses helps to explain why the HXBc2 DI mutant efficiently bound the 412d antibody but not CCR5, and thus was unable to infect CD4/CCR5 cells.

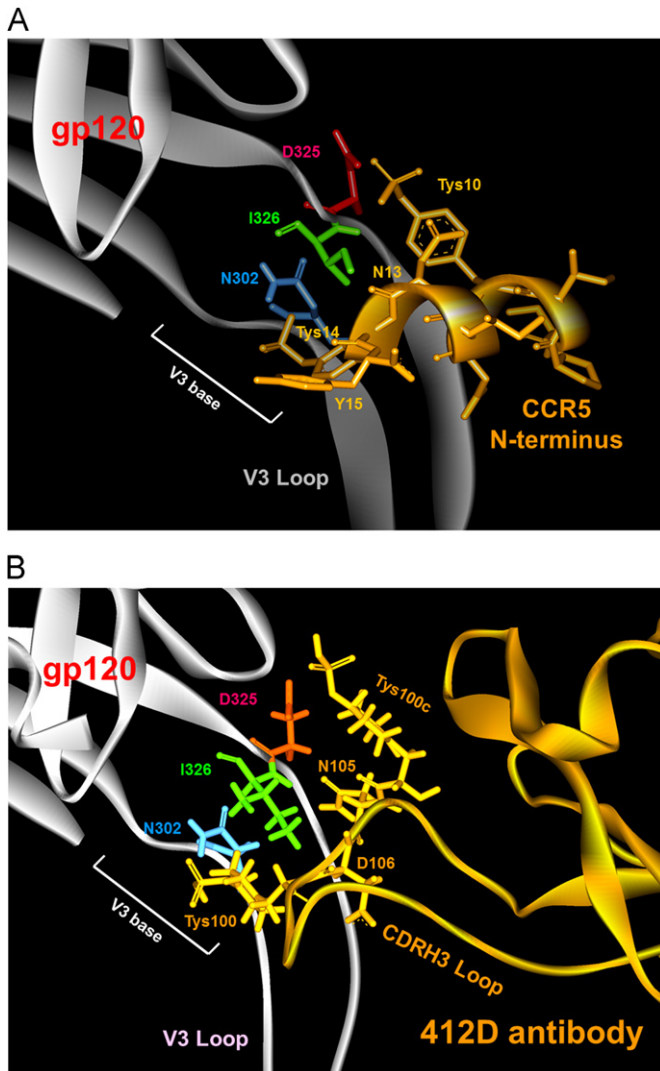
Second, our results suggest that additional interactions besides those involving the base of the gp120 V3 loop are necessary for competent CCR5 attachment. When Gln 310 and Arg 311 were deleted from the V3 tip of the DI mutant, the resulting D1ΔQR mutant gained the ability to use CCR5, exhibiting a typical dual-tropic phenotype. The deletion of residues 310 and 311 was necessary but not sufficient for CCR5 binding and R5 tropism. Apparently, the presence of Gln 310 and Arg 311 interferes with the interaction of gp120 with CCR5. Previous studies have suggested that the tip of the V3 loop may contact the second and third extracellular loops (ECL2 and ECL3) of the coreceptors (Chabot and Broder, 2000; Maeda et al., 2008b; Samson et al., 1997; Wu et al., 1997; Zhang et al., 2007). The proposed two-point contact involving the base and tip of the gp120 V3 loop with the CCR5 N-terminus and extracellular loops, respectively, implies that the length and spatial geometry of the V3 loop are both important for efficient CCR5 binding. The QR insertion in the V3 tip could conceivably alter either of these V3 parameters, disrupting the interaction with CCR5. CCR5 and CXCR4 usage



might be dictated by different residues within the V3 loop. For R5 tropism, residues 302, 325 and 326 in the V3 base, residues in the V3 tip, and the length and orientation of the V3 loop are important; for X4 tropism, other residues like 306, 321 and 322, as previously reported (Cardozo et al., 2007), appear to be important.

Our previous studies (Xiang et al., 2010) have shown that changes in the length or orientation of the V3 loop can disrupt the stability of the HIV-1 envelope glycoprotein trimer. We showed that single amino acid insertions in the V3 loop can destabilize the envelope glycoprotein trimer, and that the envelope glycoproteins of R5 viruses are more sensitive than those of X4 viruses to these insertions (Xiang et al., 2010). Some of the alterations of the V3 loop studied here affected the stability of the envelope

glycoprotein trimer, resulting in shedding of gp120 and loss of infectivity. The impact of some V3 changes on envelope glycoprotein trimer stability differed in R5, X4 and R5X4 viruses. For example, the N302D change resulted in gp120 shedding in the envelope glycoproteins of the R5 YU2 isolate, but not in the envelope glycoproteins of the X4 HXBc2 or R5X4 89.6 isolates. In our previous study (Xiang et al., 2010), insertions in the V3 loop of the X4 HXBc2 isolate did not induce shedding of gp120, whereas the same V3 changes in R5 isolates did. Our results indicate that R5X4 Envs (89.6 or HXBc2 D1AQR) resemble X4 Envs with respect to the induction of gp120 shedding by V3 changes. Together, the data indicate that R5 isolates are more prone to the shedding of gp120 as a result of the introduction of changes in the V3 loop. In



**Fig. 6.** Models of the interactions of the V3 loop with CCR5 and the 412d antibody. The interactions of the CCR5 N-terminal domain (A) or the 412d antibody (B) with the V3 loop of the HIV-1 gp120 envelope glycoprotein were modeled. Residues 302, 325 and 326 in the base of the gp120 V3 loop and their interaction with the CCR5 N-terminal peptide (A) and the 412d antibody (B) are shown. The models are based on the NMR structure of CCR5<sup>2–15</sup> (PDB ID: 2RLL) and the x-ray crystal structure of the gp120–412d–CD4 complex (PDB ID: 2QAD) (Huang et al., 2007).

the unliganded HIV-1 envelope glycoprotein trimer, the V3 loop has been suggested to project towards the trimer axis (Mao et al., 2012; Xiang et al., 2010). The V3 loops of R5 viruses may be accommodated differently in the envelope glycoprotein structures that about the trimer axis than the V3 loops of X4 and dual-tropic viruses.

Our results add to an understanding of the gp120 V3 loop determinants that dictate coreceptor usage, and provide mechanistic insights into the particular V3 changes involved in the shift from mono tropism to dual tropism. This understanding may assist attempts to block the interaction of HIV-1 with its coreceptors.

## Materials and methods

### HIV-1 envelope glycoprotein V3 loop mutants

Mutations were introduced into the pSVIIIenv plasmid expressing the full-length envelope glycoproteins from the HXBc2 HIV-1

strain (Sullivan et al., 1995), using the QuikChange II XL site-directed mutagenesis protocol (Stratagene). The presence of the desired mutations was confirmed by DNA sequencing. The gp120 substitution mutants are designated with the amino acid residue to the right of the number substituted for the amino acid residue to the left of the number. All residues are numbered according to those of the prototypic HXBc2 sequence, as per current convention (Korber et al., 1998).

### Syncytium formation assay

The ability of the HIV-1 envelope glycoprotein variants to mediate cell–cell fusion was determined by the  $\alpha$ -complementation assay (Holland et al., 2004). In this assay, the N-terminal ( $\alpha$ ) fragment of  $\beta$ -galactosidase is coexpressed with the HIV-1 envelope glycoproteins; the C-terminal ( $\omega$ ) fragment of  $\beta$ -galactosidase is expressed in the receptor-bearing target cells. Upon fusion of the envelope glycoprotein-expressing cells and the target cells, the  $\beta$ -galactosidase is reconstituted, and its activity can be measured. The assay was performed as previously described (Xiang et al., 2010). Briefly, 293 T cells in six-well plates were transfected with plasmids expressing the  $\beta$ -galactosidase  $\alpha$  fragment, the HIV-1 envelope glycoproteins and the HIV-1 Tat protein in a 4:4:1 weight ratio. Cf2Th-CD4/CCR5 cells or Cf2Th-CD4/CXCR4 cells in 100-mm dishes were transfected with 3  $\mu$ g of a plasmid expressing the  $\beta$ -galactosidase  $\omega$  fragment. On the next day, the Cf2Th target cells were lifted from the plates with 5 mM EDTA in phosphate-buffered saline (EDTA-PBS) and reseeded at  $10^5$  cells/well in 96-well plates. One day later, the transfected 293 T cells were lifted from the plates with 5 mM EDTA-PBS and diluted to  $2 \times 10^5$  cells/ml with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (DMEM-10). Approximately  $2 \times 10^4$  293 T cells (in 100  $\mu$ l) expressing the HIV-1 envelope glycoproteins were added in triplicate to each well of a 96-well plate containing the target cells. The cocultivated cells were cultured at 37 °C in a CO<sub>2</sub> incubator for 4 h. The medium was then removed, and the cells were washed once with PBS and lysed in 20  $\mu$ l of lysis buffer. The plate was covered with Parafilm, wrapped in plastic film and placed at –70 °C for 30 min. The  $\beta$ -galactosidase activity was measured with a Galacto-Star  $\beta$ -galactosidase reporter gene assay system for mammalian cells (Tropix, Bedford, MA), using a Berthold Microplate Luminometer LB 96 V (Promega).

### Infection by single round luciferase-expressing HIV-1

Envelope complementation assays were performed using HIV-1 viruses capable of only a single round of infection, as described previously (Pacheco et al., 2008). Briefly, recombinant luciferase-expressing HIV-1 viruses (Helseth et al., 1990) were generated by transfecting 293 T cells with the pSVIIIenv plasmid expressing the HIV-1 envelope glycoproteins of interest, the pCMV $\Delta$ P1 $\Delta$ envpA packaging plasmid, and an HIV-1 vector plasmid at a 1:1:3 ratio, using the calcium phosphate transfection method (Invitrogen). The HIV-1 vector plasmid expresses an RNA that can be packaged into virions, reverse transcribed, and integrated into a target cell, where it encodes firefly luciferase. Forty-eight hours after transfection, supernatants containing reporter viruses were harvested and cleared by low-speed centrifugation. The amounts of virus in the supernatants were assessed by measurement of reverse transcriptase (RT) (Rho et al., 1981). For infection, Cf2Th-CD4/CCR5 or Cf2Th-CD4/CXCR4 cells were seeded at a density of 6000 cells/well in 96-well luminometer-compatible tissue culture plates. The following day, the cells were incubated with 2500 RT units of recombinant virus per well. Two days later, the cells were lysed with 30  $\mu$ l of passive lysis buffer (Promega), and the



luciferase activity measured in the Berthold microplate luminometer LB 96 V (Promega).

### Immunoprecipitation of HIV-1 envelope glycoproteins

293 T cells were transfected with pSVIIenv plasmids expressing the HIV-1 envelope glycoproteins and a plasmid expressing the HIV-1 Tat protein. One day later, the cells were metabolically labeled for 16 h with 100  $\mu$ Ci/ml of [ $^{35}$ S]-methionine/cysteine ([ $^{35}$ S] protein labeling mix; Perkin-Elmer) in Dulbecco's modified Eagle's medium lacking methionine and cysteine and supplemented with 5% dialyzed fetal bovine serum. The cell lysates (containing gp160 and gp120) and media (containing gp120) were used for immunoprecipitation. Briefly, 100  $\mu$ l of clarified cell lysate or medium was incubated with 100  $\mu$ l of 10% protein A-Sepharose beads (Amersham Biosciences), 50  $\mu$ l of 4% bovine serum albumin, and 1  $\mu$ g of monoclonal antibody with or without sCD4 (10  $\mu$ g/ml) or, instead of the monoclonal antibody, 4  $\mu$ l of a mixture of sera from HIV-1-infected individuals. The mixtures were brought to a volume of 1 ml with PBS and incubated on a shaking platform at room temperature for 1 h. The pelleted beads were washed twice with 0.5 M NaCl-PBS and once with PBS. The beads were then suspended in a 2  $\times$  gel-loading buffer, and boiled for 3 min. Following the removal of the beads by centrifugation, the supernatants were loaded on a 10% SDS-polyacrylamide gel. The gel was fixed, dried at 80  $^{\circ}$ C for 2 h and exposed to X-ray film.

### CCR5 binding assay

To assess CCR5 binding ability, normalized amounts of radiolabeled gp120 envelope glycoproteins from transfected 293 T-cell supernatants were incubated with an excess of sCD4 and Cf2Th-CCR5 cells, as previously described (Xiang et al., 2010). Briefly, Cf2Th-CCR5 cells were lifted from the plate by using 5 mM EDTA-PBS (pH 7.5). After a wash with DMEM, the cells were resuspended in DMEM and added to 1.5-ml microcentrifuge tubes ( $10^6$  cells/tube). The radiolabeled gp120-containing cell supernatants (400  $\mu$ l) and 10  $\mu$ g of sCD4 were added to the tube, and the volume was adjusted to 1 ml with DMEM. The tubes were rocked at room temperature for 1 h. The cells were then washed once with PBS and lysed in 1 ml of buffer containing 1% NP-40. The cell lysates were precipitated with a mixture of sera from HIV-1-infected individuals and protein A-Sepharose beads (Amersham Biosciences) at 4  $^{\circ}$ C for 2 h. The precipitated gp120 was analyzed by SDS-PAGE and autoradiography.

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